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<p>Information on targets for anticancer therapy that are most likely to lead to cancer cell death and/or cessation of growth is necessary. Human melanoma cells exhibit irreversible growth arrest and terminally differentiate on treatment with recombinant human fibroblast interferon-β (IFN-beta) and a protein kinase C activator meserein (MEZ). To identify and characterize the milieu of gene expression alterations associated with growth arrest and differentiation we applied subtraction hybridization. One of the genes so identified is mda-7, a novel tumor cell specific, growth suppressor gene. Mda-7 gene expression is elevated in terminally differentiated human melanoma cells, however, ectopic expression of recombinant mda-7 initiates growth suppression and apoptosis. Interestingly this tumor growth suppression property of mda-7 is not limited to melanoma alone and is evident in diverse cancers of various origins and having different genetic alterations. Utilizing a replication deficient adenoviral vector, the tumor growth suppression/apoptosis induction property of mda-7 was studied with a view towards the development of a new experimental tumor gene therapeutic. When tested in vivo in nude mice, using both tumorigenesis and experimental gene therapy protocols, Ad.mda-7 S suppresses tumor growth and inhibits cancer progression. In contrast, no discernible biological effect is elicited by both normal human epithelial or fibroblast cells when infected with Ad.mda-7 S. The differential growth suppression activity of mda-7 towards cancer cells and its selective anti-tumor effects illustrate a novel class of cancer growth suppressor genes with great promise for targeted therapy of human cancers.</p>			
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INTRODUCTION

Abnormalities in cellular differentiation are a common incidence in cancer development and progression. Treatment of cancer cells with differentiation inducers could potentially result in reprogramming of their gene expression to that of more normal cells resulting in loss of proliferative ability and induction of terminal differentiation. Analysis of gene expression changes between cancer and normal cells is enabling our understanding of this deadly disease. Using the approach of subtraction hybridization we identified a melanoma differentiation associated gene-7, *mda-7*, that shows ubiquitous tumor growth suppression when ectopically expressed. Interestingly normal cells appear to be refractive to *mda-7* induced growth suppression. *mda-7* is a novel gene, and its expression pattern correlates with the induction of melanoma terminal differentiation. The *mda-7* cDNA encodes a polypeptide of 23.8 kDa and when ectopically expressed in tumor cells of various origins induces growth suppression. The mechanism of tumor growth suppression by *mda-7* is however an enigma. The present project aims at elucidating the mechanism of *mda-7* induced growth suppression and employs breast cancer cells as a model system and an adeno-viral expression system by which the *mda-7* gene is ectopically expressed. The research findings show that the mechanism of growth suppression by *mda-7* involves the induction of programmed cell death or apoptosis and is mediated by the pro-apoptotic protein BAX. The *in vitro* findings are validated by similar outcomes *in vivo* in nude mice. These findings suggest that *mda-7* could potentially be developed into a gene therapeutic to combat the deadly disease of breast cancer as well as a number of other types of carcinomas.

The research accomplishments of the first year of this Post-doctoral training grant are described below.

The specific aims of the proposal are in three parts and progress has been made on two out of three sections of the grant. Progress on each of the specific aims will be described separately for each section.

SPECIFIC AIM 1.

Determine the role of *mda-7* in human breast cell growth (cellular mitosis) and cancer progression, and it's relationship to p21 expression. Based on the subcellular localization of MDA-7 to the nucleus especially during cellular mitosis and its potential involvement in chromatin related mitotic events we investigated the message expression profile of *mda-7* in synchronous populations of MCF-7 and HeLa cells. Cells were synchronized using either the S-phase blocker, aphidacholine or

the M-phase blocker Nocodazole using previously published methods. Northern blotting as well as the more sensitive method of RT-PCR followed by Southern blotting analysis of total cellular RNA revealed mda-7 expression to be specifically restricted to a brief period during the cell cycle. Analyses of parallel cell populations by FACS analyses revealed the majority of the mda-7 expressing cells to be in the G2/M-phase of the cell cycle. Western blotting analyses of mitotic marker gene expression such as, Cyclin B, cdc2 and phosphorylated histone 3 revealed a corresponding increase in these proteins.

In order to be able to follow mda-7 subcellular localization during cell cycle progression Green Fluorescent Protein (GFP) mda-7 chimeric constructs of MDA-7 (MDA-7), MDA-7Δ-C(MDA-7Δ-C) and MDA-7Δ-N (MDA-7Δ-N) were generated. The two deletion constructs were constructed with a view to determine the functional domain of MDA-7. Ectopic expression of the three constructs in comparison to the GFP.Vector (GFP.V) revealed that all three mda-7 constructs to be growth suppressors to varying degrees, MDA-7Δ-C> MDA-7> MDA-7Δ-N> GFP.V. To establish subcellular localization the cells were visualized using a fluorescence microscope. GFP by itself is present throughout the cell, in the nucleus and cytoplasm. However, MDA-7 and MDA-7Δ-C were present only in the nucleus of the cells and MDA-7Δ-N was found preferentially in the cytoplasm. These results as well as earlier finding using indirect immunofluorescence reveal MDA-7 to be a nuclear protein when expressed in transformed cells. Interestingly, when MDA-7 is ectopically expressed in normal fibroblasts it showed a speckled cytoplasmic distribution. This differential localization of MDA-7 between tumor and normal cells may be responsible for its growth suppressive property in cancers. To further analyze MDA-7's putative association with chromatin, immunoprecipitation using anti-GFP antibodies followed by western blotting analyses using antibodies against phosphorylated histone 3, a specific modification of histone 3 that has gained recent recognition as a mitosis related event. Findings show that phosphorylated histone 3 is co-precipitated with MDA-7 and this interaction is mediated by the carboxy terminal region of MDA-7 (manuscript in preparation).

On going experiments at this time are evaluating the effect of mda-7 and mda-6 (p21) co-expression in HO-1 melanoma cells. Preliminary results suggest that co-expression of mda-7 (S) and p21 (AS) results in morphological differentiation of HO-1. It is yet to be tested if these morphologically differentiated cells have permanently lost their ability to grow.

Recombinant mda-7 protein is being generated using a mammalian expression system (His-tag) under the control of the EF1 α promoter in COS-7 cells. This recombinant protein will help generate high titer anti-MDA-7 polyclonal serum.

SPECIFIC AIM 2.

Define the mechanism by which mda-7 induces growth suppression and apoptosis in human cancer cells when overexpressed using recombinant Ad.mda-7 (sense) construct (Su/Madireddi et al,1998). Preliminary studies using Adeno-viral expression construct of *mda-7* and various types of tumors showed alterations in growth properties when introduced into the cell. Irrespective of tumor cell genotype all of the cells exhibited *mda-7* induced growth suppression. However, normal fibroblasts of both human and rodent origin were refractive to *mda-7* growth suppression. To determine the mechanism of *mda-7* mediated growth suppression of cancer cells we employed human breast cells, normal mammary epithelial cells (HMEC) as well as SV40 immortalized (HBL-100), and two variants of breast tumor cell lines, MCF-7 (p53 wild type) and T47D (p53 mutant).

a. Effect of Ad.mda-7 S on colony formation and induction of hypodiploidy in normal and cancer cells.

Infection of human tumor cells, including metastatic melanoma (C8161), glioblastoma multiforme (T98G), osteosarcoma (Saos 2) and carcinoma of the breast (MCF-7, T47D), cervix (HeLa), colon (SW480, WiDr and LS174T), nasopharynx (HONE-1) and prostate (DU-145 and LNCap) with 100 pfu/cell of Ad.mda-7 S reduced colony formation by >50% and inhibited growth in diverse cancers, in comparision to untreated cells or those infected with the null virus (Ad.null). To assess the infection efficiency of the virus particles, a parallel set of cultures were infected with 100 pfu/cell of Ad.beta-Gal virus. These petridishes were then stained using X-Gal and the ratio off blue cells/total cells was computed. The viral infection efficiency in all cases ranged between 98-100%. Therefore the growth inhibition and reduction in colony formation is a direct effect caused by the expression of the *mda-7* transgene. In stark contrast, no discernable effect was evident in normal human mammary epithelial, human skin fibroblast or SV40 transformed human breast epithelial cell lines (HBL-100).

b. Effect of Ad.null and Ad.mda-7 S on the growth of normal human skin fibroblasts and various human cancer cell lines

Other parameters analyzed upon ectopic expression of Ad.mda-7 S were alterations in cell cycle progression as assayed by fluorescence activated cell scanning (FACS). Interestingly, FACS data indicated a marked increase in hypodiploid cell populations in cancer cells infected with Ad.mda-7 S and not in the case of Ad.null or Ad.beta-Gal infected cells. No visible levels of hypodiploidy were apparent in normal human mammary

epithelial, human skin fibroblast or SV40 transformed human breast epithelial cell lines (HBL-100) infected with Ad.mda-7 S. This data strongly suggests that ectopic expression of mda-7 in cancer cells in inducing programmed cell death or apoptosis. To further this line of investigation, cell samples were analyzed after infection with Ad.mda-7 S for, inter-nucleosomal DNA degradation by DNA ladder assay, DNA cleavage by TUNNEL assay, and an early indicator of apoptosis being changes in phospholipid localization in the cell membrane by annexin V assay. All of the above parameters indicated apparent induction of apoptosis in tumor cells infected with Ad.mda-7 S.

c. Immunoblotting analyses of BAX expression in normal and breast carcinoma cells infected with Ad.mda-7 S

To dissect the molecular components involved in mda-7 initiated apoptosis some of the better characterized players of apoptosis were analyzed such as, the Bcl-2 gene family members. Of the various Bcl-2 gene family proteins analyzed by Western blotting of whole cell proteins only one of the pro-apoptotic factors, BAX was consistently induced and its induction is coincident with the expression of mda-7 and occurrence of apoptosis. No evidence for an increase in BAX protein levels were evident in normal control cells. In contrast to BAX, Bcl-2 and Bcl-XL and the viral homologue Ad.E1B are anti-apoptotic and promote cell survival by dimerizing with free BAX. A comparison of BAX:Bcl-2 ratios revealed significantly higher ratios in cancer cells infected with Ad.mda-7 S compared to normal control cells, implicating BAX as a major player in mda-7 induced cell death. The anti-apoptotic effect of Bcl-2 is mediated by the formation of a stable hetero-dimeric complex between BAX and Bcl-2 or BAX and Ad.E1, thereby nullifying the apoptotic inducing effect of BAX. Since high levels of Bcl-2 or Ad.E1B can counter the pro-apoptotic signal from BAX and BAX appears to be a major contributor to mda-7 induced cell death, studies were performed to determine if free BAX is essential for mda-7 induced cell death. Bcl-2 and Ad.E1B were ectopically expressed in two breast carcinoma cells (MCF-7 and T47D) to determine whether the cells will now be refractile to mda-7 induced apoptosis. In both instances overexpression of Ad.mda-7 S (100 pfu/cell) did not induce growth suppression and the cells were now resistant to apoptosis.

These studies provide additional evidence for mda-7 induced growth suppression of cancer cells and the activation of the BAX mediated apoptotic pathway in a tumor cell specific manner.

e. In vivo inhibition of human tumor growth in nude mice.

The next logical analyses following successful *in vitro* analyses of a growth suppressor gene, no less one which is specific for cancer

cells calls for *in vivo* analysis in animal model systems of its applicability as an anti-cancer agent in this case, a cancer gene therapeutic. Here too the Ad.mda-7 S was exploited as a gene delivery system. Two lines of investigation were carried out:

(i) The 'Tumorigenicity Model', where human breast carcinoma cells (MCF-7) which were infected with 100 pfu of Ad.mda-7 S or Ad.null 96 hr prior to implantation in nude mice. In the control group implanted with MCF-7 infected with Ad.null, tumors developed in all groups within 7 days and grew progressively during the course of the experiment (4 weeks). In stark contrast, the experimental group implanted with MCF-7 cells infected with Ad.mda-7 S exhibited statistically ($p < 0.01$) significant suppression of tumor development, as defined by tumor volume and weight.

(ii) A more stringent test for potential anti-tumor activity is the '*in vivo* Therapy Model'. Here, HeLa cells were first implanted in nude mice and tumors were allowed to grow until they reached an average volume of 150-200 mm³. The treatment regimen consisted of 3 weekly intratumoral injection of Ad.mda-7 S or Ad.null (1×10^8 pfu/injections) over 4 sites for 3 weeks followed by an additional one week observation. The experiment was terminated due to the extreme size of the tumors in the control group (Ad.null). However, statistically significant ($p < 0.05$) inhibition of tumor growth was observed in the experimental group (Ad.mda-7 S) compared to the control group. These *in vivo* results are the first evidence suggesting that mda-7 has great potential as cancer gene therapeutic.

f. Conclusions

The melanoma differentiation model system combined with subtraction hybridization has enabled the identification of differentiation induced gene products. Screening of the DISH library permitted the isolation of the mda-7 cDNA, a novel tumor growth suppressor gene. The selective ability of mda-7 to induce programmed cell death in cancer cells of diverse origins and not in normal cells (via a BAX mediated pathway) places mda-7 in a new class of tumor growth suppressors. This cell death induction by mda-7 when tested in animal model systems resulted in tumor growth inhibition and encouraging prognosis. Based on these findings mda-7 may prove to be of great value for targetted applications as an anti-cancer therapeutic of human cancers.

SUMMARY

- Ectopic expression of Ad.mda-7 S causes growth suppression in tumor and not in normal breast cells.
- mda-7 induces inter-nucleosomal DNA cleavage in human breast cancer cells.

- mda-7 induced apoptosis involves Bax and is inhibited by Bcl-2 and Adenovirus E1B.
- Pre-clinical gene therapy studies with Ad. mda-7 S and human tumors in a nude mice model shows tumor regression.
- MDA-7 is a chromatin-associated protein and is expressed specifically during cellular mitosis in normal and tumor cells.
- Histone 3 when phosphorylated during mitosis associates with MDA-7 protein.
- Ectopic expression of mda-7 may be inducing premature chromosome condensation/premature mitosis in tumor cells and thereby causing apoptotic cell death.

PUBLICATIONS AND PRESENTATIONS

Madireddi M.T , Su ZZ, , Goldstein NI, Young CSH , and Fisher PB. (1999). Past and Present Achievements in Cancer Gene Therapy. Mda-7, a novel cancer gene therapeutic for the treatment of cancers. Gene Therapy, Ed. N. Habib. Oxford Press. (In Press)

Su ZZ.*, Madireddi M.T.* , Lin JJ, Young CSH, Kitada S, Reed JC, Goldstein NI, Fisher PB (1998). The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. Proc. Nat. Acad. Sci, USA. 95:14400-14405. * equal authors

Madireddi M.T, Su Z-z., Young C.S.H.,, Goldstein N. I., and Fisher P.B. MDA-7, a novel mitotic protein that induces apoptosis in cancer cells. Keystone Symposium, Apoptosis and Cell Death, Brekenridge, CO, April, 6-11, 1999.

ENCLOSURES: Reprint

The cancer growth suppressor gene *mda-7* selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice

(melanoma differentiation associated gene 7/programmed cell death/recombinant adenovirus/selective antitumor activity)

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ABSTRACT A differentiation induction subtraction hybridization strategy is being used to identify and clone genes involved in growth control and terminal differentiation in human cancer cells. This scheme identified melanoma differentiation associated gene-7 (*mda-7*), whose expression is up-regulated as a consequence of terminal differentiation in human melanoma cells. Forced expression of *mda-7* is growth inhibitory toward diverse human tumor cells. The present studies elucidate the mechanism by which *mda-7* selectively suppresses the growth of human breast cancer cells and the consequence of ectopic expression of *mda-7* on human breast tumor formation *in vivo* in nude mice. Infection of wild-type, mutant, and null p53 human breast cancer cells with a recombinant type 5 adenovirus expressing *mda-7*, Ad.*mda-7* S, inhibited growth and induced programmed cell death (apoptosis). Induction of apoptosis correlated with an increase in BAX protein, an established inducer of programmed cell death, and an increase in the ratio of BAX to BCL-2, an established inhibitor of apoptosis. Infection of breast carcinoma cells with Ad.*mda-7* S before injection into nude mice inhibited tumor development. In contrast, ectopic expression of *mda-7* did not significantly alter cell cycle kinetics, growth rate, or survival in normal human mammary epithelial cells. These data suggest that *mda-7* induces its selective anticancer properties in human breast carcinoma cells by promoting apoptosis that occurs independent of p53 status. On the basis of its selective anticancer inhibitory activity and its direct antitumor effects, *mda-7* may represent a new class of cancer suppressor genes that could prove useful for the targeted therapy of human cancer.

Abnormalities in cellular differentiation are common occurrences during cancer development and progression (1, 2). Correction of these defects resulting in the reversion of tumor cells to a more-normal differentiated phenotype represents a potentially useful therapeutic strategy (1, 2). Although the mechanism underlying cancer growth suppression and terminal differentiation is unknown, it is hypothesized that these changes result from the activation of genes negatively regulating cell growth and the suppression of genes promoting the cancer phenotype (1, 2). Induction of terminal differentiation can occur with and without the initiation of programmed cell death (2). Identification of the genes mediating these phenomena should provide mechanistic insights into these pro-

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cesses and also may elucidate potentially novel and selective targets for cancer therapy.

Induction of terminal differentiation combined with the molecular approach of subtraction hybridization, differentiation induction subtraction hybridization, is permitting the identification of critical gene changes associated with and controlling induction of terminal differentiation in human melanoma cells (1, 3, 4–6). The combination of recombinant human fibroblast interferon (interferon β) and the antileukemic compound mezerein elicits an irreversible loss of proliferation and induces terminal differentiation in human melanoma cells (7, 8). Several melanoma differentiation-associated (*mda*) genes have been isolated that either correlate with or directly influence human melanoma cell growth and differentiation (1, 3, 4–6). These include the cyclin-dependent kinase inhibitor p21, identified as *mda-6*, *waf-1*, *cip-1*, *sdi-1* (4, 5, 9), and several novel genes, including *mda-7* and *mda-9* (10, 11).

Partial screening of a human melanoma temporally spaced differentiation inducer-treated cDNA (differentiation induction subtraction hybridization) library identified the *mda-7* cDNA consisting of 1,718 bp that encode a novel protein of 206 amino acids with a predicted M_r of 23,800 (1, 3, 11). Induction of growth arrest and terminal differentiation in human melanoma cells results in elevated expression of *mda-7* (1, 3). Moreover, the level of *mda-7* expression inversely correlates with human melanoma progression, with highest levels found in actively proliferating normal melanocytes and lowest levels in metastatic melanoma (3). Ectopic expression of a transfected *mda-7* gene in H0-1 human melanoma cells suppresses growth without inducing terminal differentiation, suggesting that this gene is involved in growth control and indirectly contributes to the terminal differentiation process (3). Additionally, ectopic expression of a transfected *mda-7* gene induces growth suppression and a reduction in colony formation in cancer cell lines of diverse origin with multiple genetic defects (11). In contrast, no overt biological response is engendered in normal human epithelial or fibroblast cells by ectopic over-expression of *mda-7* (11).

The present studies investigate the mechanism by which *mda-7* selectively inhibits the proliferation of breast cancer cells and not normal mammary epithelial cells. Evidence is presented documenting a strong correlation between ectopic expression of *mda-7* and induction of apoptosis in breast

Abbreviations: pfu, plaque-forming unit; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; HMEC, human mammary epithelial cells; HMC, high molecular weight.

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cancer cells. This process is characterized by an up-regulation of the proapoptotic effector Bax and an increase in the BAX/BCL-2 protein ratio (12, 13). A direct effect of *mda-7* "gene therapy" on the growth of human tumor xenografts in nude mice also is demonstrated. On the basis of the selective breast cancer growth inhibitory properties of *mda-7* and its apparent ability to distinguish and spare normal cells from growth inhibition and apoptosis, the *mda-7* cDNA represents a potentially effective antitumor agent for breast cancer gene therapy.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Growth and β -Galactosidase Assays. MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-453, and T47D human breast carcinoma cell lines were obtained from the American Type Culture Collection and were cultured as recommended. Normal human breast epithelial cells included immortal HBL-100 (American Type Culture Collection) and early passage mammary epithelial cells [human mammary epithelial cells (HMEC), passage nos. 9–12] (Clonetics, San Diego). HMEC cells were grown in serum-free defined medium supplied by Clonetics. To study the effect of *mda-7* on monolayer colony formation or cell growth, cells were infected with 100 plaque-forming units (pfu)/cell of Ad.*mda-7* S, Ad.Vec, or Ad. β -gal, and colony formation (3 to 4 weeks) or cell growth (daily over a 14-day period, with a medium change at days 4, 7, and 10) was determined (3, 11). To evaluate the effect of *mda-7* coexpression with Bcl-2 or Ad E1B, MCF-7 or T47D cells were transfected with 10 μ g of an *mda-7* gene cloned in a pMAM-neo vector (3), alone or in combination with 10 μ g of a Bcl-2 (pSFFV-Bcl-2) (14) or an Ad E1B (pCMV.E1B) (15) expression vector by using the lipofectin method (11). *In situ* β -galactosidase assays were performed by using standard protocols (16).

Construction and Assaying of Recombinant Adenoviruses. The recombinant replication-defective Ad.*mda-7* S was created in two steps, as described for Ad.*mda-7* antisense (11). Production of infectious virus in 293 cells, analysis of recombinant virus genomes to confirm the recombinant structure, plaque purification, and titration of virus were performed as described (17).

Cell Cycle Analysis. Fluorescence-activated cell sorter analysis was performed as described (18). The percentage of cells in the various phases of the cell cycle was estimated manually by gating the G₁/G₀, S, and G₂/M regions of the histograms. The percentage of cells to the left of the G₁/G₀ region (the A₀ region), representing apoptotic cells containing less than a diploid content of DNA, also was estimated by gating the appropriate region of the histograms.

DNA Extraction, Fragmentation Assay, and Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay. DNA was extracted, and fragmentation assays were performed as described (18) 2 and 4 days after infection of cells with 100 pfu/cell of Ad.*mda-7* S or Ad.Vec. A modified TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) method (19) was used to evaluate apoptosis in cells treated for the fragmentation assay.

Immunohistochemistry, Immunoprecipitation, and Western Blotting. These assays were performed as described (3, 11, 20–22). However, immunoreactivity in Western blotting assays was detected by enhanced chemiluminescence (ECL) (Amer sham).

Tumor Studies. MCF-7 cells were infected with 100 pfu/cell of Ad.*mda-7* S or Ad.Vec, were incubated at 37°C for 96 hr, were resuspended at 2.5 \times 10⁶ cells/ml in PBS, and were mixed 1:1 with Matrigel (Collaborative Research), and 400 μ l of this suspension (1 \times 10⁶ per animal) was injected s.c. into nude mice (Taconic Farms) (16, 23). Four weeks after injection,

animals were killed, and the tumors were removed, were snap frozen in liquid nitrogen, and were weighed. Data are presented as tumor weight. In addition, a tumor volume ratio was calculated. This is an index of tumor progression over the course of a study (23).

RESULTS

***mda-7* Selectively Induces Apoptosis in Human Breast Cancer Cells with Different p53 Genotypes.** To define a potential mechanism by which *mda-7* induces its selective effect on cancer versus normal cells and to define potential therapeutic applications for *mda-7*, we constructed replication-defective adenoviruses expressing *mda-7* (Ad.*mda-7* S) or, as a control, the β -galactosidase (Ad. β -gal) gene. Infection of human breast cancer cells, including MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-453, and T47D with 100 pfu/cell of Ad.*mda-7* S reduced growth and colony formation in comparison with untreated cells or cultures infected with a recombinant Ad lacking the *mda-7* gene Ad.CMV null (Ad.Vec) (Figs. 1 and 2 and data not shown). Infection of the same cell types with 100 pfu/cell of Ad. β -gal resulted in β -galactosidase expression in the majority of treated cells and no significant change in growth properties (Figs. 1 and 2 and data not shown). Because the different breast cancer cell lines contain either wild-type p53 (MCF-7), mutant p53 (MDA-MB-231, MDA-MB-453, and T47D), or null p53 (MDA-MB-157), these results document that the growth-inhibitory activity of *mda-7* occurs independently of the mode of action of this extensively studied tumor suppressor gene that is frequently altered in human cancers.

In contrast to malignant breast tumor cells, infection of immortal normal human breast epithelial cells, HBL-100, with 100 pfu/cell of Ad.*mda-7* S resulted in a similar kinetics of growth and cloning efficiency in liquid medium as found after infection with Ad.Vec or Ad. β -gal (Fig. 1). Unaltered growth kinetics was also evident in early passage normal HMEC infected with the three viruses (Fig. 1). These results extend previous observations by using the less efficient approach of DNA transfection, indicating that ectopic expression of *mda-7* selectively inhibits the growth of breast cancer cells *in vitro* (11).

A consistent observation with many tumor cells infected with Ad.*mda-7* S is a change in cellular and nuclear morphology suggestive of programmed cell death. Fluorescence-activated cell sorter analyses of DNA content were performed to determine the effects of *mda-7* on apoptosis-associated DNA degradation and to explore whether alterations in cell cycle progression occur. In all of the breast cancer cell lines, a hypodiploid (A₀) peak appeared or increased after infection with Ad.*mda-7* S relative to infection with Ad.Vec (data not shown). This putative apoptotic response was not evident after infection of normal human breast cells, HBL-100 or HMEC, with 100 pfu/cell of Ad.*mda-7* S.

Infection of MCF-7 and T47D cells with 10 or 100 pfu/cell of Ad.*mda-7* S resulted in a temporal induction of growth suppression and apoptosis as indicated by the formation of cells with a hypodiploid DNA content, nucleosomal DNA ladders, positive TUNEL (TdT-mediated dUTP nick end labeling) reaction, and positive annexin V staining (Figs. 1, 3, and 4C and data not shown). When MCF-7 and T47D cells were analyzed for MDA-7 protein by using indirect immunofluorescence with MDA-7-specific mAbs 2 days after infection with 100 pfu/cell of Ad.*mda-7* S, intense nuclear staining was visible (Fig. 4A and data not shown). In contrast, none of the parameters indicative of apoptosis occurred in HMEC or HBL-100 cells after infection with 100 pfu/cell of Ad.*mda-7* S. The absence of an effect in normal breast cells did not result from a failure to infect these cell types and express genes controlled by the CMV promoter (as indicated by β -galacto-

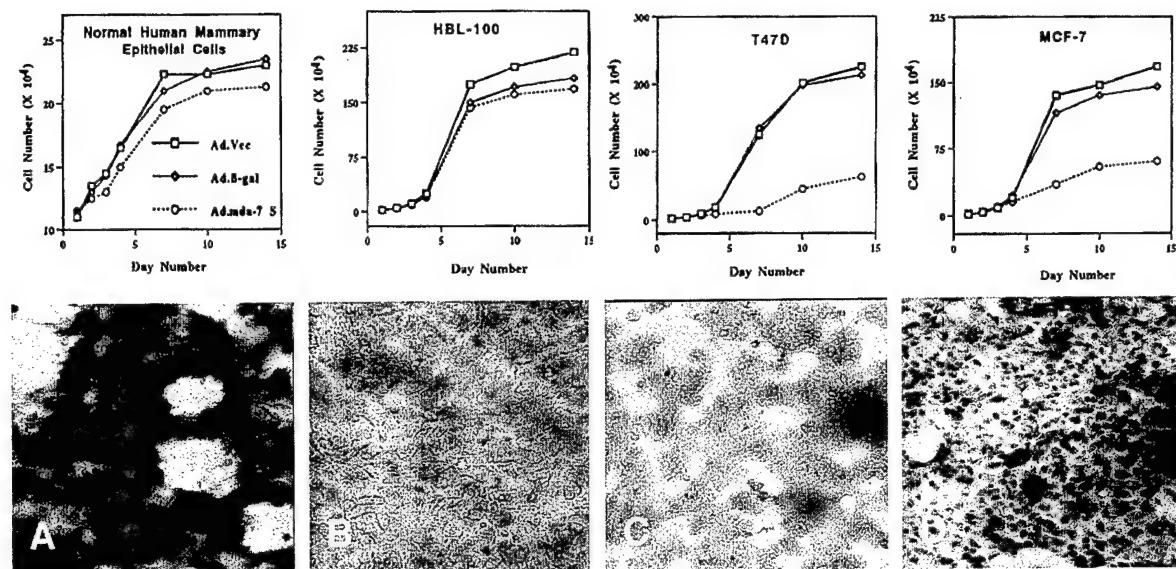


FIG. 1. Effect of Ad. *Vec*, Ad.β-gal, and Ad.*mda-7* S on the growth of normal breast and breast cancer cells. The indicated cell types were infected with 100 pfu/cell of Ad. *Vec*, Ad.β-gal, or Ad.*mda-7* S, and cell growth was determined over a 14-day period. Triplicate samples varied by $\leq 10\%$. Similar results ($\pm 15\%$) were obtained in two additional replicate studies. In the lower panels, β -galactosidase activity was assayed in HMEC (A), HBL-100 (B), T47D (C), and MCF-7 (D) cells 48 hr after infection with the Ad.β-gal.

sidase staining after infection with Ad.β-gal) or to produce the *MDA-7* protein (Figs. 1 and 5). Immunoprecipitation analysis of [³⁵S]methionine-labeled cell lysates from MCF-7, T47D, HBL-100, and HMEC cells with *MDA-7* mAb indicated the presence of equivalent amounts of both the predicted *MDA-7* protein (≈ 23.8 kDa) and a high molecular weight (HMC) interacting protein (≈ 90 – 110 kDa) in MCF-7, T47D, and HBL-100 cells and reduced levels of both proteins in HMEC cells (Fig. 5). These findings establish that *mda-7* can induce apoptosis differentially in breast carcinomas but not in normal breast epithelial cells. Moreover, this selective apoptotic-inducing effect is not a direct consequence of differential levels of the *MDA-7* protein or the HMC interacting protein in breast cancer versus normal breast epithelial cells.

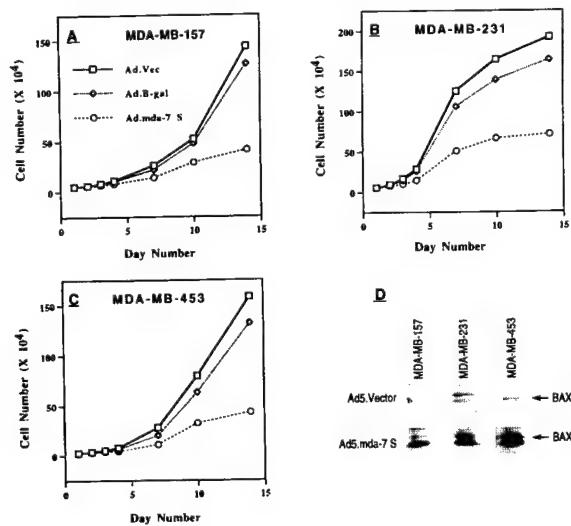


FIG. 2. Effect of Ad. *mda-7* S on growth and BAX protein levels in human breast cancer cells. The experimental growth protocol was as described in the legend to Fig. 1. The breast carcinoma cell lines analyzed include MDA-MB-157 (A), MDA-MB-231 (B), and MDA-MB-453 (C). D provides immunoblot analyses of BAX expression 2 days after infection of the indicated breast cancer cell line with 100 pfu/cell of Ad. *Vec* or Ad.*mda-7* S. Coomassie blue staining of gels indicated equal protein loading.

Induction of Apoptosis in Human Breast Cancer Cell Lines Correlates with an Elevation in BAX Levels. Programmed cell death reflects a balance between signaling events and molecules that either promote or inhibit apoptosis (12, 13, 24, 25). Current data support the hypothesis that the ratio of death antagonists to agonists determines whether a cell will respond to apoptotic signals. Proteins such as Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, and Ad E1B (19 and 55 kDa) protect cells from specific programs of apoptosis whereas BAX, Bad, Bak, and Bcl-X_S proteins stimulate apoptosis in specific target cells (12, 13, 24, 25). We, therefore, determined by Western blotting if induction of *mda-7*-induced apoptosis in human breast carcinoma cells altered the expression of specific proteins associated with

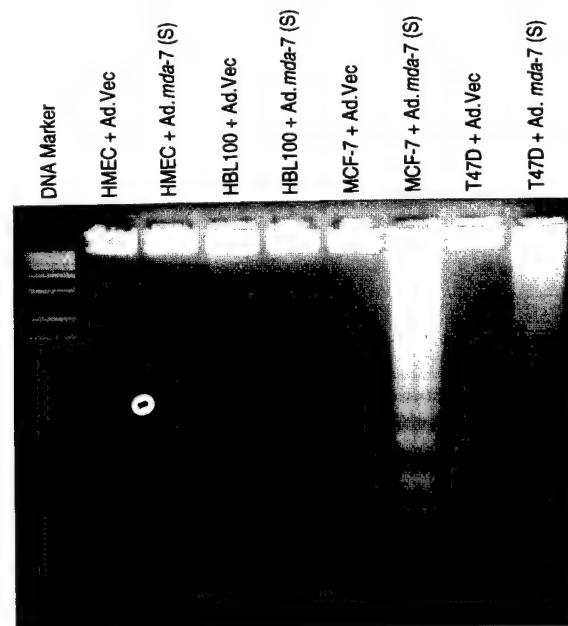


FIG. 3. Induction of nucleosomal DNA degradation in human breast cancer cells, but not in normal breast epithelial cells, infected with Ad.*mda-7* S. The indicated cell types were infected with 100 pfu/cell of Ad. *Vec* or Ad.*mda-7* S and were analyzed for nucleosomal DNA degradation 4 days after infection.

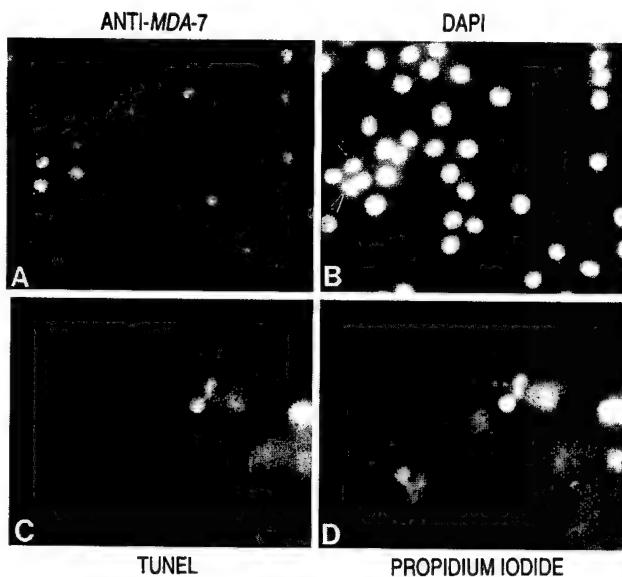


FIG. 4. Nuclear localization of *mda-7* and induction of a positive TUNEL reaction in MCF-7 cells infected with Ad. *mda-7* S. MCF-7 cells were doubly stained with Anti-*MDA-7* antibody (A) and 4',6-diamidino-2-phenylindole (DAPI) (B) 2 days after infection with 100 pfu/cell of Ad.*mda-7* S. The position of two mitotic cells stained with Anti-*mda-7* antibody are shown in A, and the corresponding 4',6-diamidino-2-phenylindole counterstain is indicated in B (arrows label metaphase chromosomes). MCF-7 cells 4 days after infection with 100 pfu/cell of Ad.*mda-7* S were doubly stained by the TUNEL method (C) and propidium iodide (D).

apoptosis (Figs. 2D and 6). Western blotting of lysates prepared 2 and 4 days after infection of HMEC, HBL-100, MCF-7, and T47D cells with 100 pfu/cell of either Ad.*mda-7* S or Ad.*Vec* demonstrated increased expression of BAX in both MCF-7 (p53 wild-type protein) and T47D (p53 mutant protein) cells after infection with Ad.*mda-7* S but not with Ad.*Vec* (Fig. 6A). Ad.*mda-7* S infection of MCF-7 and T47D cells also resulted in elevated levels of processed BAX protein of 18–21 kDa (25). Up-regulation of BAX after infection with Ad.*mda-7* S was also evident in additional breast carcinoma cell lines containing mutant p53 (MDA-MB-231 and MDA-MB-453) or null p53 (MDA-MB-157) (Fig. 2D). Comparison of BAX to BCL-2 protein ratios revealed BAX/BCL-2 to be significantly higher in breast cancer cells (Fig. 6B and data not

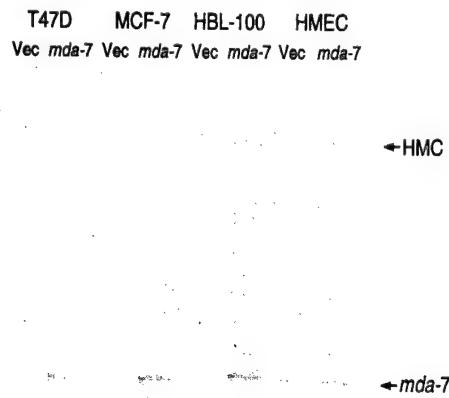
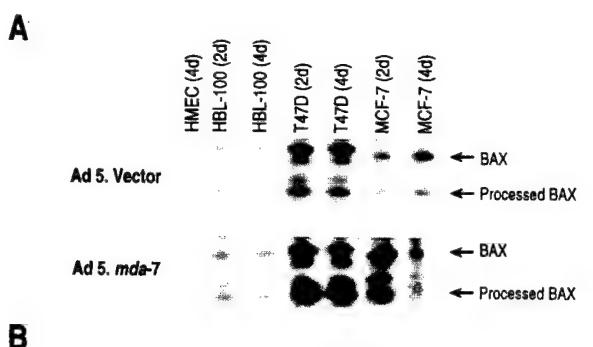


FIG. 5. Immunoprecipitation of *MDA-7* and an HMC protein with an *MDA-7* mAb. The indicated cell lines were infected with 100 pfu/cell of Ad.*Vec* or Ad.*mda-7* for 4 days and were labeled with [³⁵S]methionine, and the levels of the *MDA-7* and HMC proteins were determined by immunoprecipitation analysis. Coomassie blue staining of gels indicated equal protein loading.



^aCells were infected with 100 pfu/cell of Ad.*Vec* or Ad.*mda-7* S and cell lysates were prepared after 4 days and analyzed by Western blotting using antibodies to the indicated proteins.
^bRelative expression levels indicated by + and - designations, with ++ maximum and +/- minimum expression. A - designation indicates no detectable protein.

FIG. 6. Expression of Bcl-2, Bax, and Bak in normal breast epithelial and breast carcinoma cells infected with Ad. *mda-7* S or Ad.*Vec*. (A) Immunoblot analyses of BAX protein in normal mammary epithelial and cancer cells. Equal amounts of whole cell protein (verified by Coomassie blue staining) from 2- and 4-day cell cultures infected with 100 pfu/cell of Ad.*Vec* or Ad.*mda-7* S were resolved by SDS/PAGE (4–20%), were immunoblotted, and were probed with Anti-BAX mAb. Note that both intact BAX and processed BAX proteins are visible in the breast cancer cell lines 2 days after Ad.*mda-7* infection. Low levels of BAX in 4-day MCF-7 is caused by proteolytic degradation because the cells in this study were 70% apoptotic by 4 days after infection with Ad.*mda-7* S. (B) Tabular compilation of protein levels of the Bcl-2 gene family members, BCL-2 and BAK, 4 days after infection with 100 pfu/cell of Ad.*mda-7* S or Ad.*Vec* in normal breast epithelial cells and breast carcinoma cell lines.

shown), implicating BAX as a potential component in *mda-7*-induced programmed cell death. Ectopic expression of *mda-7* in HBL-100 cells variably modified BAX expression, which is generally quantitatively less than that observed in breast cancer cells. Moreover, the ratio of BAX/BCL-2 was consistently lower in HBL-100 cells after infection with Ad.*mda-7* S than in the breast cancer cells. In contrast, in early passage HMEC, representing the best approximation of normal breast epithelial cells, Ad.*mda-7* S failed to induce the BAX protein. No consistent changes were seen in other apoptosis-modifying proteins, including BAK, BAD, BAG-1, and BCL-X, after infection of breast carcinoma or normal breast epithelial cells with Ad.*mda-7* S (Fig. 6B and data not shown). On the basis of these observations, it appears that BAX may be a crucial regulator of apoptosis induced selectively in breast cancer versus normal mammary epithelial cells after ectopic overexpression of *mda-7*.

Overexpression of Bcl-2 and Ad E1B proteins protects cells from apoptosis induced by diverse stimuli (13, 24, 25). This effect may be mediated by the formation of a stable complex between BAX and BCL-2 or BAX and Ad E1B proteins by heterodimerization, thereby nullifying the apoptotic-inducing effect of BAX (26–29). Because high levels of the antiapoptosis proteins BCL-2 or Ad E1B can counteract the proapoptotic signaling of BAX, studies were performed to determine whether overexpression of Bcl-2 or Ad E1B would protect breast carcinoma cells from *mda-7*-induced growth suppression and apoptosis. Cotransfection of MCF-7 and T47D cells with a pMAMneo-*mda-7* expression construct (permitting controlled expression of *mda-7* by dexamethasone and containing a neomycin resistance gene permitting colony selection in G418) (3) and a pSFFV-bcl-2 expression construct (express-

ing Bcl-2 and containing a neomycin resistance gene permitting colony selection in G418) (14) or pCMV.E1B expression vector (expressing both Ad E1B proteins and containing a neomycin resistance gene permitting colony selection in G418) (15) rescued cells from the growth-inhibitory effect of *mda-7* (Fig. 7). Moreover, MCF-7 cells, engineered to stably overexpress Bcl-2, were refractory to *mda-7*-induced (100 pfu/cell) growth suppression and apoptosis (data not shown). These results provide additional evidence that *mda-7* induces growth suppression and apoptosis in breast cancer cells by inducing a programmed cell death pathway that can be modified directly by overexpressing the antiapoptotic proteins BCL-2 or Ad E1B.

Ectopic Expression of *mda-7* in Human Breast Carcinoma Cells Inhibits Tumor Development in Nude Mice. On the basis of *in vitro* studies indicating selective growth-inhibitory and apoptosis-inducing effects of *mda-7* when overexpressed in cancer cells, we investigated the effect of ectopic expression of *mda-7* on tumor formation by breast cancer cells *in vivo* in nude mice. The breast carcinoma cell line MCF-7 was infected with 100 pfu/cell of either Ad.*mda-7* S or Ad.*Vec* 96 hr before implantation in nude mice. In the control Ad.*Vec*-infected cells, tumors developed in all groups within 7 days and grew progressively during the course of the experiment (4 weeks). In contrast, the group of animals injected with tumor cells infected with Ad.*mda-7* S exhibited a statistically significant ($P < 0.01$) suppression of tumor development, as defined by tumor volume and tumor weight (Fig. 8 and data not shown). Moreover, Ad.*mda-7* S also inhibited the growth of MCF-7 tumors initiated in nude mice (100–150 mm³) before a 3-week Ad.*mda-7* S therapy protocol (three weekly intratumoral injections of 1×10^8 pfu/injection in 100 μ l over four sites) (data not shown). These experimental findings document that

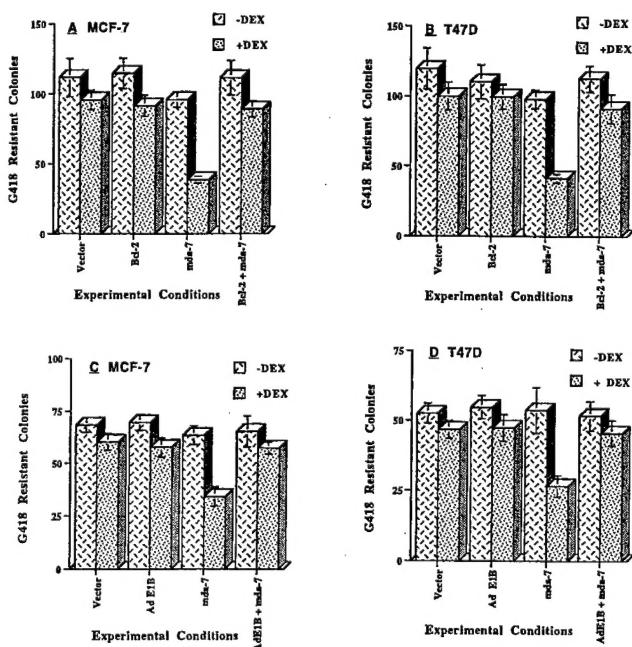


FIG. 7. Effect of inducible *mda-7* expression alone and in combination with Bcl-2 or Ad E1B expression on colony formation in MCF-7 and T47D cells. Cells were transfected with a pMAMneo-*mda-7* and a pSFFV-bcl-2 [MCF-7 (A) or T47D (B)] or a pCMV.E1B [MCF-7 (C) and T47D (D)] expression plasmid, alone and in combination, and were grown in medium containing 300 μ g of G418 per milliliter and in the presence or absence of 1×10^{-6} M dexamethasone (DEX). Colonies were enumerated after \approx 3–4 weeks of growth \pm SD for five replicate plates. Similar results have been obtained \pm 15% in a replicate study (data not shown).

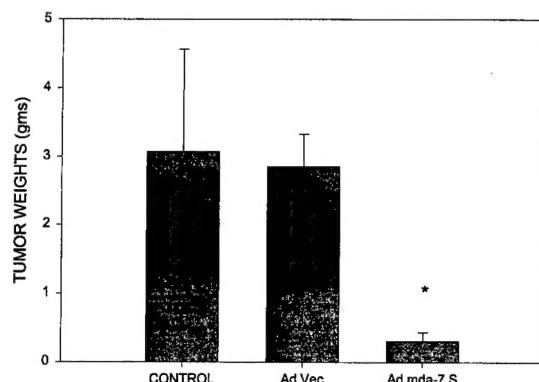


FIG. 8. Effect of *mda-7* on the growth of MCF-7 cells in nude mice. MCF-7 cells were uninfected (CONTROL) or infected with 100 pfu/cell of Ad.*Vec* or Ad.*mda-7* S and were incubated for 4 days at 37°C. Cells were removed with trypsin, were mixed with Matrigel (1:1), and were injected into nude mice (10^6 cells/animal). Results are shown as tumor weight in grams \pm SD. Statistical significance was determined with a Student's *t* test using the computer program SIGMA STAT (Jandel, San Rafael, CA), and a *P* value of <0.05 is indicated by an asterisk. Two additional tumorigenicity studies have been performed with qualitatively similar results (data not shown).

Ad.*mda-7* S can inhibit tumor formation and progression directly *in vivo*.

DISCUSSION

In the present studies, we describe the antitumor and apoptosis-promoting properties of a cancer growth suppressor gene, *mda-7*. On the basis of the previously confirmed genetic defects in the human tumor cell lines analyzed, including mutations in p53 and/or RB, it is evident that the biological activity of *mda-7* does not depend on the action of these tumor suppressor genes (11). The capacity of a cancer suppressor gene, such as *mda-7*, to efficiently inhibit the growth of wild-type, mutant, and null p53 breast carcinoma cells, as well as cancer cells with additional defects (11), supports the intriguing possibility, confirmed by *in vivo* studies, that *mda-7* may prove efficacious in the gene-based therapy of human breast and other cancers. Moreover, because elevated expression of *mda-7* in normal cells does not elicit a deleterious effect, a problem often encountered when using conventional gene therapy approaches should be avoided.

The mechanism by which *mda-7* differentially inhibits growth and induces apoptosis in breast cancer versus normal mammary epithelial cells remains to be determined. Preliminary studies using mAbs specific for *mda-7* demonstrate an association of this protein with chromatin in cells undergoing mitosis (data not shown). Moreover, in human melanoma cells, induction of terminal differentiation by treatment with interferon β plus mezerein results in translocation of *mda-7* from the cytosol into the nucleus of differentiated cells. These two observations suggest that *mda-7* may be associated with chromatin remodeling, which is apparent during both mitosis and differentiation. On the basis of primary protein sequence analysis, *mda-7* does not appear to possess any nuclear localization signals, suggesting that the MDA-7 protein requires an association with a cytosolic chaperone to translocate into the nucleus (3, 11). In this context, identification of proteins that interact with *mda-7* (such as the HMC) (3, 11) and that facilitate migration into the nucleus may provide insights into the mechanism by which *mda-7* selectively suppresses malignant but not normal mammary epithelial cell growth.

The Bcl-2 gene family members are important genetic elements in maintaining homeostasis between cell survival and death. The present study demonstrates that *mda-7*-induced

apoptosis in breast cancer cells is associated with the up-regulation of the proapoptotic protein BAX whereas levels of BCL-2, BCL-X_L, BAK, BAD, and BAG-1 were unchanged. The observation that the cell death signal induced by *MDA-7* can be counteracted by overexpression of Bcl-2 or its viral homologue adenovirus E1B (12, 27, 28) is consistent with a prominent role for *MDA-7*-induced up-regulation of BAX in the apoptotic mechanism used by this tumor suppressor. In this regard, recent studies suggest that BAX also may function as a tumor suppressor (29–31).

Although BAX expression is regulated positively by wild-type p53 (27), the ability of *mda-7* to induce BAX is clearly p53-independent, suggesting that alternative pathways can be involved in BAX up-regulation after ectopic overexpression of *mda-7*. At present, it is unknown whether *mda-7* directly or indirectly induces BAX expression in cancer cells. However, given the lack of similarity of *mda-7* to any known transcription factors, we suspect that it at least requires other cofactors. The lack of a significant biological effect of *mda-7* in normal cells may involve a failure of *MDA-7* protein to accumulate in the nucleus and induce the appropriate gene expression changes, such as Bax, that are necessary to inhibit cell growth and/or induce apoptosis or a combination of these effects. These possibilities are amenable to experimental testing. Defining the molecular basis of action of *mda-7* is merited and should provide important insights into the role of this cancer growth suppressor gene in tumor development and progression. This information also should assist in exploiting *mda-7* for cancer therapy.

Ectopic expression of *mda-7* by using a recombinant adenovirus delivery approach induces growth suppression and apoptosis in human breast cancer cell lines *in vitro* and inhibits human breast tumor growth *in vivo* in nude mice. In contrast, normal breast epithelial cells do not exhibit analogous changes in cell growth or survival after ectopic overexpression of *mda-7*. These findings indicate that *mda-7* represents a class of cancer-specific growth-arresting and apoptosis-inducing genes that may prove efficacious for the targeted therapy of breast cancer.

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The cancer growth suppressor gene *mda-7* selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice

(melanoma differentiation associated gene 7/programmed cell death/recombinant adenovirus/selective antitumor activity)

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ABSTRACT A differentiation induction subtraction hybridization strategy is being used to identify and clone genes involved in growth control and terminal differentiation in human cancer cells. This scheme identified melanoma differentiation associated gene-7 (*mda-7*), whose expression is up-regulated as a consequence of terminal differentiation in human melanoma cells. Forced expression of *mda-7* is growth inhibitory toward diverse human tumor cells. The present studies elucidate the mechanism by which *mda-7* selectively suppresses the growth of human breast cancer cells and the consequence of ectopic expression of *mda-7* on human breast tumor formation *in vivo* in nude mice. Infection of wild-type, mutant, and null p53 human breast cancer cells with a recombinant type 5 adenovirus expressing *mda-7*, Ad.*mda-7* S, inhibited growth and induced programmed cell death (apoptosis). Induction of apoptosis correlated with an increase in BAX protein, an established inducer of programmed cell death, and an increase in the ratio of BAX to BCL-2, an established inhibitor of apoptosis. Infection of breast carcinoma cells with Ad.*mda-7* S before injection into nude mice inhibited tumor development. In contrast, ectopic expression of *mda-7* did not significantly alter cell cycle kinetics, growth rate, or survival in normal human mammary epithelial cells. These data suggest that *mda-7* induces its selective anticancer properties in human breast carcinoma cells by promoting apoptosis that occurs independent of p53 status. On the basis of its selective anticancer inhibitory activity and its direct antitumor effects, *mda-7* may represent a new class of cancer suppressor genes that could prove useful for the targeted therapy of human cancer.

Abnormalities in cellular differentiation are common occurrences during cancer development and progression (1, 2). Correction of these defects resulting in the reversion of tumor cells to a more-normal differentiated phenotype represents a potentially useful therapeutic strategy (1, 2). Although the mechanism underlying cancer growth suppression and terminal differentiation is unknown, it is hypothesized that these changes result from the activation of genes negatively regulating cell growth and the suppression of genes promoting the cancer phenotype (1, 2). Induction of terminal differentiation can occur with and without the initiation of programmed cell death (2). Identification of the genes mediating these phenomena should provide mechanistic insights into these pro-

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cesses and also may elucidate potentially novel and selective targets for cancer therapy.

Induction of terminal differentiation combined with the molecular approach of subtraction hybridization, differentiation induction subtraction hybridization, is permitting the identification of critical gene changes associated with and controlling induction of terminal differentiation in human melanoma cells (1, 3, 4–6). The combination of recombinant human fibroblast interferon (interferon β) and the antileukemic compound mezerein elicits an irreversible loss of proliferation and induces terminal differentiation in human melanoma cells (7, 8). Several melanoma differentiation-associated (*mda*) genes have been isolated that either correlate with or directly influence human melanoma cell growth and differentiation (1, 3, 4–6). These include the cyclin-dependent kinase inhibitor p21, identified as *mda-6*, *waf-1*, *cip-1*, *sdi-1* (4, 5, 9), and several novel genes, including *mda-7* and *mda-9* (10, 11).

Partial screening of a human melanoma temporally spaced differentiation inducer-treated cDNA (differentiation induction subtraction hybridization) library identified the *mda-7* cDNA consisting of 1,718 bp that encode a novel protein of 206 amino acids with a predicted M_r of 23,800 (1, 3, 11). Induction of growth arrest and terminal differentiation in human melanoma cells results in elevated expression of *mda-7* (1, 3). Moreover, the level of *mda-7* expression inversely correlates with human melanoma progression, with highest levels found in actively proliferating normal melanocytes and lowest levels in metastatic melanoma (3). Ectopic expression of a transfected *mda-7* gene in H0-1 human melanoma cells suppresses growth without inducing terminal differentiation, suggesting that this gene is involved in growth control and indirectly contributes to the terminal differentiation process (3). Additionally, ectopic expression of a transfected *mda-7* gene induces growth suppression and a reduction in colony formation in cancer cell lines of diverse origin with multiple genetic defects (11). In contrast, no overt biological response is engendered in normal human epithelial or fibroblast cells by ectopic over-expression of *mda-7* (11).

The present studies investigate the mechanism by which *mda-7* selectively inhibits the proliferation of breast cancer cells and not normal mammary epithelial cells. Evidence is presented documenting a strong correlation between ectopic expression of *mda-7* and induction of apoptosis in breast

Abbreviations: pfu, plaque-forming unit; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; HMEC, human mammary epithelial cells; HMC, high molecular weight.

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mda-7 Induces Apoptosis in Cancer Cells and Initiates Premature Chromosome Condensation

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A differentiation induction subtraction hybridization approach enabled the cloning of melanoma differentiation associated gene-7, (*mda-7*). Ectopic expression of *mda-7* results in growth inhibition of various types of cancer cells. However, normal cells are resistant to over expression of *mda-7* and do not exhibit marked alterations in growth properties¹. To elucidate the tumor growth suppression properties of *mda-7* we employed a recombinant type 5 adenovirus expressing *mda-7*, Ad.*mda-7* S. and human breast cancer and normal breast epithelial cells. Infection of breast cancer cells of various genotypes with Ad.*mda-7* S induced growth arrest and programmed cell death both, *in vitro* and *in vivo*. *Mda-7* induced cell death is independent of the cellular p53 status. Interestingly, Ad.*mda-7* S expression corresponds with downstream elevation in BAX protein levels, and induction of cell death is inhibited by Bcl-2 co-expression, implicating BAX as a major factor in *mda-7* induced apoptosis². Monoclonal antiserum generated against a *MDA-7* peptide reveal nuclear localization of the *MDA-7* protein. HeLa cells undergoing mitosis reveal mitotic chromatin staining with anti-*MDA-7* antibody which is similar to that of core-histones, and is seen as early as pre-prophase up to telophase, implicating *MDA-7* in a mitosis-related function. Recent evidence suggests that H3 phosphorylation (PH3) at the N-terminal tail reduces their affinity for DNA and facilitates the movement of nucleosomes and access to chromatin by condensation factors, transcription factors and proteases activated during apoptotic cell death³. Preliminary data using an anti-PH3 antibody in immunoprecipitation assays reveals that recombinant *MDA-7*-GFP co-precipitates with PH3, while truncations of the N- and C-terminal regions of *MDA-7*-GFP do not appear to form stable immunocomplexes with PH3. Taken together the immunofluorescence and precipitation data suggest that *MDA-7* may be involved in chromatin remolding which would ultimately lead to chromatin compaction during mitosis. It is tempting to suggest that, inappropriate expression of *mda-7* using Ad.*mda-7* S in cancer cells may be inducing premature chromosome condensation (PCC) and cell death.

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